D,L-*S*-Methyllipoic Acid Methyl Ester, a Kinetically Viable Model for S-Protonated Lipoic Acid as the Oxidizing Agent in Reductive Acyl Transfers Catalyzed by the 2-Oxoacid Dehydrogenase Multienzyme Complexes†

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Received July 28, 1997; Revised Manuscript Received November 5, 1997

ABSTRACT: D,L-*S*(6,8)-Methyllipoic acid methyl ester triflate salt (D,L-*S*-methyllipoic acid methyl ester) was synthesized as a model for *S*-protonated lipoic acid, suggested to be the active form of lipoic acid in the reductive acylation catalyzed by the E1 and E2 enzymes of the 2-oxoacid dehydrogenase multienzyme complexes by a previous model [Chiu, C. C., Chung, A., Barletta, G., and Jordan, F. (1996) *J. Am. Chem. Soc. 118*, 11026−11029]. While in that earlier study lipoic acid could only trap only the enamine/C2α-carbanion intermediate in an intramolecular model, and with the assistance of a mercury compound to shift the equilibrium to the products, D,L-*S*-methyllipoic acid methyl ester could trap the enamine derived from 2-α-methoxybenzyl-3,4,5-trimethylthiazolium salt in an intermolecular reaction in the absence of a mercury compound, and with a rate constant of 6.6×10^4 M^{-1} s^{-1}. A tetrahedral adduct at the C2α-position formed between the enamine and D,L-*S*-methyllipoic acid methyl ester was isolated and characterized. The reaction likely takes place by two-electron nucleophilic attack, since no evidence was found for C2α-linked homodimers, expected from a free-radical mechanism. The results suggest that, in the reductive acyl transfer, there is nucleophilic attack by the enamine at one of the sulfur atoms of the lipoic acid [probably at S8, according to Frey, P. A., Flournoy, D. S., Gruys, K., and Yang, Y. S. (1989) *Ann. N.Y. Acad. Sci. 373*, 21−35], while there is concomitant electrophilic catalysis by a proton juxtaposed at S6 via a general acid catalyst located on the E1 enzyme. Oxidation of the enamine derived from C2α-hydroxybenzyl-3,4,5-trimethylthiazolium salt by D,L-*S*-methyllipoic acid methyl ester was also deduced on the basis of the formation of 2-benzoylthiazolium ion as a major product; however, the tetrahedral intermediate could not be detected. Oxidation of the enamine by D,L-*S*-methyllipoic acid methyl ester can take place with either an ether or an alcohol at the C2α position of the enamine.

The 2-oxoacid dehydrogenase multienzyme complexes comprise a family of enzymes with great importance in carbohydrate and amino acid metabolism (*1*). The smallest substrate for such enzymes is pyruvate which is converted to acetylcoenzyme-A by the pyruvate dehydrogenase multienzyme complex (PDHc)[1] (see review in ref *2*). The reactions proceed by thiamin diphosphate (ThDP)-catalyzed decarboxylation of the substrate to C2α-hydroxyethylidene•-ThDP, which is variously called an enamine or C2α-carbanion. For a number of years, our group has studied the structure and reactivity of this C2α-carbanion/enamine intermediate: (1) Conditions were found for generation and spectroscopic characterization of the intermediate, the results of which strongly suggest a highly conjugated planar structure, emphasizing the enamine resonance contribution (*3*); (2) The acid−base properties and the kinetics of protonation−deprotonation at the C2α position were studied both in DMSO (*4*) and in aqueous solutions (*5*, *6*); (3) Oxidation of the enamine was examined, first under electrochemical conditions (*7*), and later under the influence of isoalloxazine as an FAD surrogate (*8*) and lipoic acid ethyl ester (*9*). These reactions correspond to elementary steps involving the enamine intermediate, respectively, in protonation on the nonoxidative decarboxylation pathways (performed by pyruvate decarboxylase and benzoylformate decarboxylase) and oxidation of the enamine by pyruvate oxidase (requires FAD, in addition to ThDP) and by the variety of 2-oxoacid dehydrogenase multienzyme complexes (requiring covalently-bound lipoic acid in addition to ThDP, see Scheme 1).

Two mechanisms have been put forth to explain reductive acylation of lipoyl−E2 by the enamine−E1 complex (see Scheme 1 and ref *10* for review): (1) formation of a tetrahedral adduct by nucleophilic attack of the C2α carbanion at one of the two sulfur atoms of lipoic acid leading to ring opening, followed by collapse of the adduct to acyldihydrolipoamide and free ThDP; and (2) electron and proton transfer without formation of a tetrahedral intermediate, leading to 2-acylThDP−E1 and dihydrolipoyl−E2, followed by acyl transfer from ThDP−E1 to dihydrolipoyl−

† Supported by NIH Grant GM-50380, the Rutgers University Busch Grant, and Roche Diagnostics Systems, Inc., Somerville, NJ.

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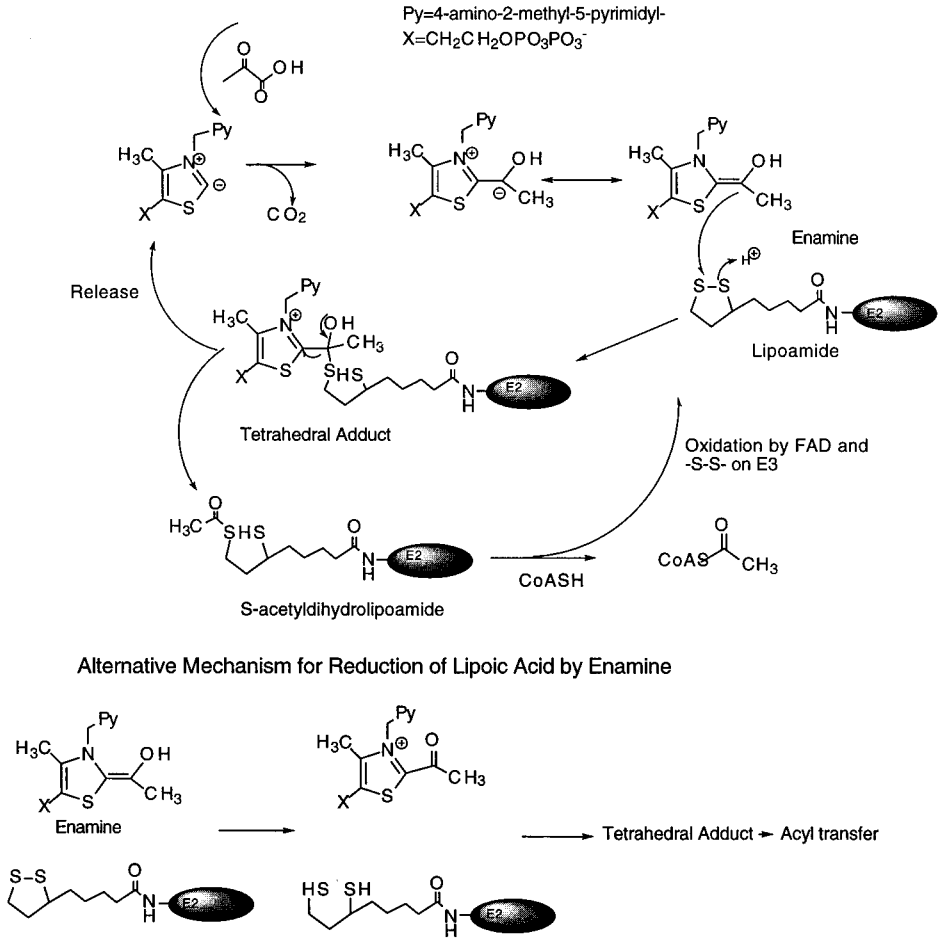
[1] Abbreviations: PDHc, pyruvate dehydrogenase multienzyme complex; E1, pyruvate dehydrogenase component of PDHc; E2, dihydrolipoamide acetyltransferase component of PDHc; ThDP, thiamin diphosphate; ES-MS, electron-spray mass spectrometry; D,L-*S*-methyllipoic acid methyl ester, mixture of the triflate salt of the S6- and S8-methylated analogs of D,L-lipoic acid methyl ester; DBU, 1,8-diazabicyclo[5,4,0]undec-7-ene; FAD, flavin adenine dinucleotide.

S0006-2960(97)01835-7 CCC: $15.00
Published on Web 02/03/1998

Scheme 1: Mechanism for Reductive Acetylation of Lipoamide−E2 by the Enamine on E1 in PDHc



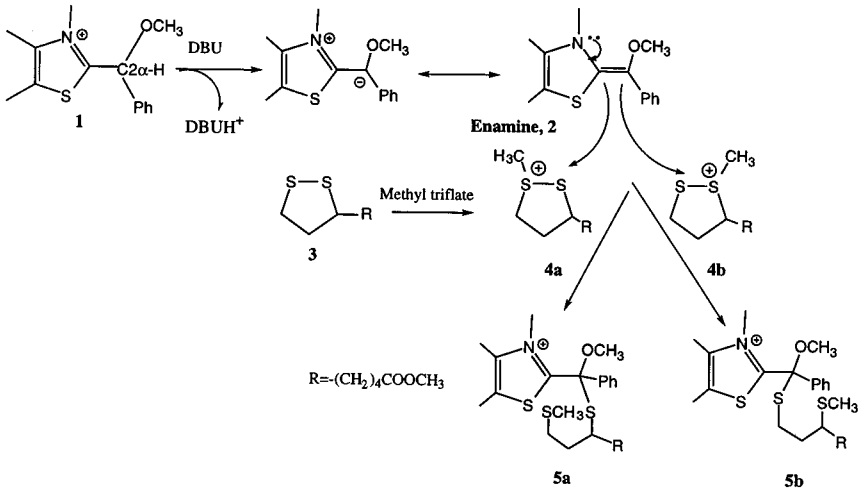
E2. The principal difference between the two alternative mechanisms is that the redox reaction between the enamine and lipoic acid takes place prior to acyl transfer in the latter, and concomitant with formation of the tetrahedral adduct in the former.

Earlier attempts have been made to mimic the reaction in chemical models (*11*−*14*). A recent report from this laboratory presented an intramolecular system comprising lipoic acid and C2α-hydroxybenzylthiazolium salts or C2α-methoxybenzylthiazolium salts (*9*), and the reaction was triggered by the addition of a base to generate the enamine intermediate in situ. The reaction was imperceptibly slow until a mercury trapping agent (C_6H_5HgCl) was added to the reaction mixture, and unless the redox partners were incorporated in an intramolecular system. Even under those conditions, the reaction was very sluggish, estimated to take place at least one million times slower than the enzymatic turnover. That study suggested that the presence of an electrophile juxtaposed on the disulfide of lipoic acid would solve the enamine-trapping problem on the enzyme (these enzymes are certainly not believed to depend on Hg for their activities), so as to shift the equilibrium to the product side. A general acid catalyst would be the most likely candidate to accomplish this, but this we could not mimic since lipoic acid is susceptible to polymerization reactions under both acidic and alkaline conditions. Also, the enamine in our model is generated under basic conditions, conditions that are inconsistent with S-protonation of lipoic acid. Therefore, a methyl group was selected to mimic the proton, assuming that the *S*-methyllipoic acid resembles the S-protonated form in its reactivity. This model may then also lead to identification of the putative tetrahedral intermediate formed between the C2α carbon and one of the sulfur atoms of lipoic acid. Herein is reported the first successful isolation and characterization of the tetrahedral adduct in an intermolecular model system (Scheme 2) consisting of a C2α-methoxybenzylthiazolium salt as the enamine precursor and the mixture of the triflate salt of the S6- and S8-methylated analogs of D,L-lipoic acid methyl ester (abbreviated as D,L-*S*-methyllipoic acid methyl ester, **4**). The enamine was found to react with D,L-*S*-methyllipoic acid methyl ester very rapidly, measurable only with a stopped-flow spectrophotometer. The reaction between a C2α-hydroxybenzylthiazolium salt and D,L-*S*-methyllipoic acid methyl ester in the presence of a base gave 2-benzoylthiazolium ion as the major product, indicating that the enamine could be oxidized by this thiosulfonium salt with either an ether or an alcohol at the C2α-position.

EXPERIMENTAL PROCEDURES

Materials. C2α-Methoxybenzyl-3,4,5-trimethylthiazolium triflate (**1**) and C2α-hydroxybenzyl-3,4,5-trimethylthiazolium triflate (**6**) were synthesized from 4,5-dimethylthiazole (Pyrazine Specialties, Inc.) by following previously described procedures (*7*), except for the use of methyl triflate for nitrogen quaternization instead of trimethyloxonium fluoroborate. The two thiazolium salts were also synthesized enriched with ^{13}C at the C2α-position using the same procedure, but starting with [7-^{13}C]benzaldehyde (Isotec, Inc.,

Scheme 2: Proposed Mechanism for the Reaction of the Enamine Derived from **1** with D,L-*S*-Methyllipoic Acid Methyl Ester



Miamisburg, OH). D,L-lipoic acid methyl ester was synthesized from D,L-lipoic acid (Fluka). The mixture of the triflate salt of the S6- and S8-methylated analogs of D,L-lipoic acid methyl ester (abbreviated as D,L-*S*-methyllipoic acid methyl ester) (**4**) was prepared according to Ravenscroft et al. (*15*). Other chemicals were from the sources indicated and were used without further purification unless otherwise noted: 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU), anhydrous THF, CH_2Cl_2, DMSO, and methyl triflate from Aldrich; Silica Gel (200−400 mesh), methanol, petroleum ether, ethyl ether, and CH_2Cl_2 from Fisher Scientific.

Instrumentation. ^{13}C NMR studies were carried out on a Varian VXR-500S spectrometer. Chemical shifts were calibrated against the solvent peak of DMSO-d_6 or the TMS peak in $CDCl_3$. Electron-spray mass spectra (ES-MS) were obtained from PeptidoGenic Research & Co. Inc. (Livermore, CA). The stopped-flow measurements were carried out on a Hi-Tech PQ/SF-53 instrument.

^{13}C NMR Studies. [C2α-^{13}C]C2α-Methoxybenzyl-3,4,5-trimethylthiazolium triflate (**1**) (4.1 mg) was dissolved in 0.75 mL of DMSO-d_6, and the spectrum was recorded after purging the solution with Ar for 3 min. Next a 2-fold molar excess of DBU (3.1 μL) was added to the tube, and the contents were mixed by shaking; then the spectrum was re-recorded. Finally, a 2.2-fold molar excess (over **1**) of D,L-*S*-methyllipoic acid methyl ester (**4**) (8.8 mg) was added, and after mixing the spectrum was re-recorded.

[C2α-^{13}C]C2α-Hydroxybenzyl-3,4,5-trimethylthiazolium triflate (**6**, 9.8 mg) was dissolved in 0.75 mL of DMSO-d_6, and the spectrum was recorded after a 3-min Ar purge. The spectrum was re-recorded after addition of 5.7 μL (1.5-fold molar excess) of DBU. Finally, the spectrum was re-recorded after addition of D,L-*S*-methyllipoic acid methyl ester (**4**) (19.6 mg, 2.0-fold molar excess over **6**).

*Synthesis and Isolation of the Tetrahedral Adduct between the Enamine from **1** and **4***. To a solution of D,L-lipoic acid methyl ester (77 mg, 0.35 mmol) in 2 mL of dry CH_2Cl_2 purged with Ar for 3 min was added slowly methyl triflate (62 μL, 0.39 mmol). The reaction was stirred for 0.5 h until the pale yellow color disappeared. TLC (CH_2Cl_2/MeOH, 20:1) showed that all of the methyl lipoate was converted to the *S*-methyl derivative (solution A). In a second flask, to a solution of C2α-methoxybenzyl-3,4,5-trimethylthiazolium triflate (139 mg, 0.35 mmol in 2 mL of CH_2Cl_2 purged with Ar for 3 min) was added DBU (78.5 μL, 0.53 mmol) slowly, and the solution turned dark yellow (solution B). After mixing solutions A and B, the color of solution B was bleached. After 5 min, a drop of trifluoroacetic acid was added to quench the reaction. The crude product was purified by flash column chromatography with a gradient of 3, 5, 6, and 7% MeOH in CH_2Cl_2 (50 mL of each). After the first 100 mL was eluted, 9 mL fractions were collected. Fractions 2, 3, and 4 contained 131 mg of product (59.3% yield based on **1**). In a different experiment, the same procedure was followed and the reaction mixture was directly applied to the LC−ES-MS to help identify *all* components in the reaction mixture.

*Reaction of C2α-Hydroxybenzyl-3,4,5-trimethylthiazolium triflate with **4***. To a solution of C2α-hydroxybenzyl-3,4,5-trimethylthiazolium triflate (24.9 mg, 0.065 mmol) in 1 mL of dry CH_2Cl_2 purged with Ar for 3 min was added DBU (14.6 μL, 0.097 mmol), and the solution contents were mixed for a few min. The orange solution was added to another flask containing D,L-*S*-methyllipoic acid methyl ester (25 mg, 0.065 mmol) and purged with Ar. After stirring for a few minutes, trifluoroacetic acid (25 μL, 0.325 mmol) was added to terminate the reaction. A sample was collected without further purification for ES-MS.

Measurement of Rates. C2α-Methoxybenzyl-3,4,5-trimethylthiazolium triflate (5.34 mg) was dissolved in 300 μL of anhydrous DMSO. A 5.4 μL portion of this solution was diluted into 4 mL of DMSO in the stopped-flow solution chamber, resulting in a 0.0600 mM concentration of **1**.

Fifteen microliters of DBU was dissolved in 0.5 mL of DMSO and 12 μL of this solution was added to the syringe containing **1** ([DBU] = 0.600 mM). The solution was purged with Ar for 5 min. The second syringe contained 0.600 mM D,L-*S*-methyllipoic acid methyl ester in DMSO (diluted from 30 μL of a stock solution of 15.44 mg dissolved in 500 μL of DMSO). The VIS absorption was detected at 383.7 nm (ϵ = 15 000). Since equal volumes were mixed on the stopped-flow, the final concentrations were one-half of those quoted. Pseudo first-order rate constants were obtained from exponential fitting of the VIS absorbance change at 383.7 nm using the software supplied by HI-TECH Instruments.

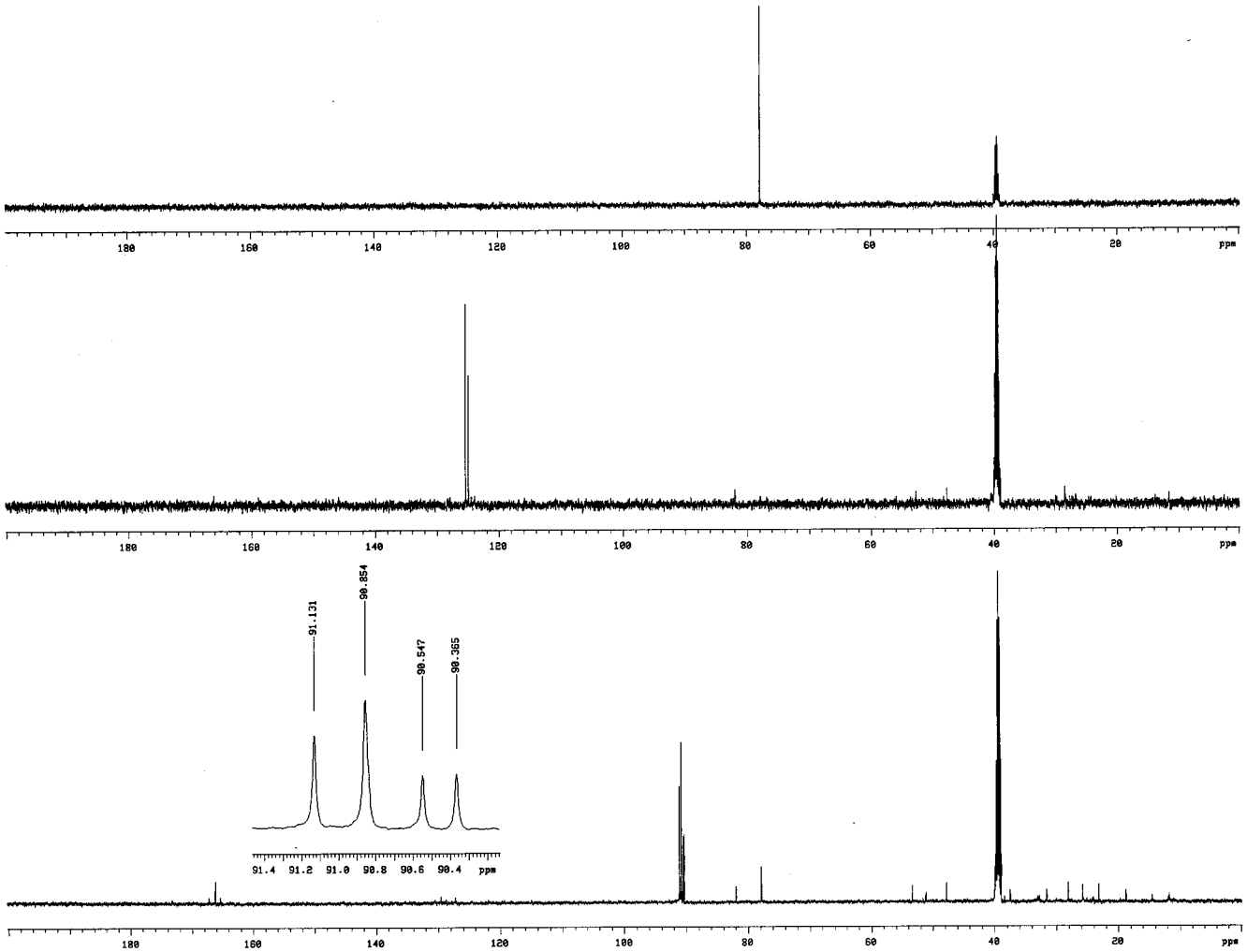


FIGURE 1: ^{13}C NMR spectrum of [2α-^{13}C]**1** in the absence and presence of base and D,L-*S*-methyllipoic acid methyl ester. Top: 4.1 mg of [2α-^{13}C] **1** in DMSO-d_6; middle: C2α of **2** after adding 2 equiv (3.1 μL) of DBU; bottom: C2α of **5a** and **5b** after adding 2.2 equiv (8.8 mg) of **4**.

RESULTS

*Characterization of the Putative Tetrahedral Adduct between the Enamine from **1** and **4**.* Several questions needed to be addressed concerning this novel oxidizing agent **4**. First, before one can interpret the kinetic results, the product of the reaction between the enamine and **4** had to characterized. As is shown in Figure 1, the ^{13}C chemical shift of the C2α carbon in **1** is at 77.860 ppm. After addition of a 2-fold molar excess of DBU, the original resonance disappeared and two new resonances at δ 125.396 and 124.927 ppm appeared, indicating that the starting material was converted to the enamine, the two resonances corresponding to the (*E*) and (*Z*)-configurations of the enamine (*3*), strongly supporting the enamine resonance structure over the carbanion one. Subsequent addition of a 2.2-fold molar excess of D,L-*S*-methyllipoic acid methyl ester resulted in the disappearance of the enamine resonances and the appearance of four new resonances at δ 91.131, 90.854, 90.547, and 90.365 ppm. The presence of four resonances can be rationalized if they pertain to two regioisomers **5a** and **5b**, while the C2α carbon of each regioisomer is also diastereotopic vs the chiral center on lipoic acid. The average deshielding compared to the starting material is 12.86 ppm, but the chemical shift is still in the range expected for tetrahedral carbon, with the additional deshielding due to the sulfur atom.

Next, the putative intermediate was isolated and purified. Detection of a positive ion with $m/e = 248.2$ in the ES-MS spectrum of **1** indicated that only the positive ion component of the salt was being detected in the positive ion mode. The peak at $m/e = 235.2$ in the ES-MS of D,L-*S*-methyllipoic acid methyl ester **4** also corresponds to the mass of the positive ion, and the absence of higher molecular mass peaks indicated that no polymerization of lipoate had taken place. Consistent with reports from Caserio and co-workers, the polymerization of lipoate can be prevented by methylating one of the sulfur atoms. The formation of the tetrahedral adduct **5a** and **5b** was suggested by the ES-MS experiment of the new product isolated from the reaction of the enamine derived from **1** with D,L-*S*-methyllipoic acid methyl ester. As shown in Table 1, the addition of two starting materials with positive ion masses of 248.2 for **1** (the *m/e* for the enamine derived from it should be 247.2 on account of the loss the C2α-H) and 235.2 for **4** produced the tetrahedral adduct with a positive ion mass of 482.3 (exactly as expected for 247.2 + 235.2). Considering the total yield of 59.3%, the tetrahedral adduct can be regarded as the major and most stable product of the reaction.

Table 1: Summary of ES-MS Data on Compound **1** Reacted with D,L-*S*-Methyllipoic Acid Methyl Ester after Addition of DBU

compound	formula/calcd mass	mass detected (*m*/*e*)
1	$C_{14}H_{18}ONS$/248.4	248.2
4	$C_{10}H_{19}O2S_2$/235.4	235.2
5a, **5b**	$C_{24}H_{36}O_3NS_3$/482.7	482.3

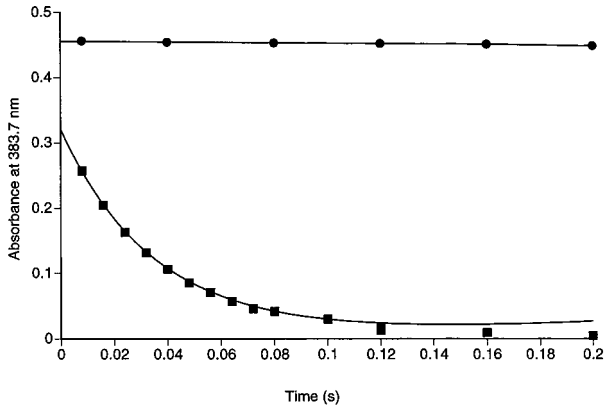


FIGURE 2: Stopped-flow kinetic traces for oxidation of the enamine from **1** by D,L-*S*-methyllipoic acid methyl ester. The enamine generated by addition of excess DBU to 0.030 mM C2α-methoxybenzyl-3,4,5-trimethylthiazolium was mixed with and oxidized by 0.300 mM D,L-*S*-methyllipoic acid methyl ester in DMSO at 27.0 °C. The flat line is the control, showing the stability of the enamine in the absence of the oxidizing agent.

In a duplicate experiment (using identical conditions and concentrations), an aliquot was removed from the reaction mixture and applied directly to the LC−ES-MS. In the positive ion mode, peaks corresponding (*m*/*e*) to $DBUH^+$ (153.0), **1** (observed at 248.2, calculated at 248.4), **4** (observed at 235.2, calculated at 235.4), and **5a** and **5b** (observed at 482.3, calculated at 482.7) were observed. There was no evidence for a dimer formed from two enamines, as would be expected had the reaction taken place by a stepwise electron transfer pathway with a cation radical intermediate (such C2α-linked homodimers were identified as products of the electrochemical oxidation of the enamine which had been shown to proceed by a single-electron mechanism; see ref *7*). The peak corresponding to the dimer of the cation radical derived from **1** should have appeared at $m/e = 247.4$ (since it is a dication, it should be detected at an m/e = one-half the mass for the entire dication). Within experimental error, none of this product was present.

*Kinetic Studies of the Enamine from **1** with **4**.* The oxidation of the enamine (generated by the addition of DBU to **1**) by D,L-*S*-methyllipoic acid methyl ester was monitored on a stopped-flow instrument. As can be seen in Figure 2, in the absence of D,L-*S*-methyllipoic acid methyl ester, the VIS absorption at 383.7 nm (the λ_{max} of the enamine) is stable with time. Once a 10-fold excess of D,L-*S*-methyllipoic acid methyl ester was added, more than 80% of the enamine was oxidized in less than 0.2 s. Five solutions of D,L-*S*-methyllipoic acid methyl ester at 10, 15, 20, 25, and 30 times the enamine concentration were used to enable estimation of a second-order rate constant. A plot of the pseudo first-order k_{obs} against [D,L-*S*-methyllipoic acid methyl ester] was linear (Figure 3), providing a second-order rate constant of $6.6 \times 10^4\ M^{-1}\ s^{-1}$.

Reaction of C2α-Hydroxybenzyl-3,4,5-trimethylthiazolium Triflate with D,L-S-Methyllipoic Acid Methyl Ester in the

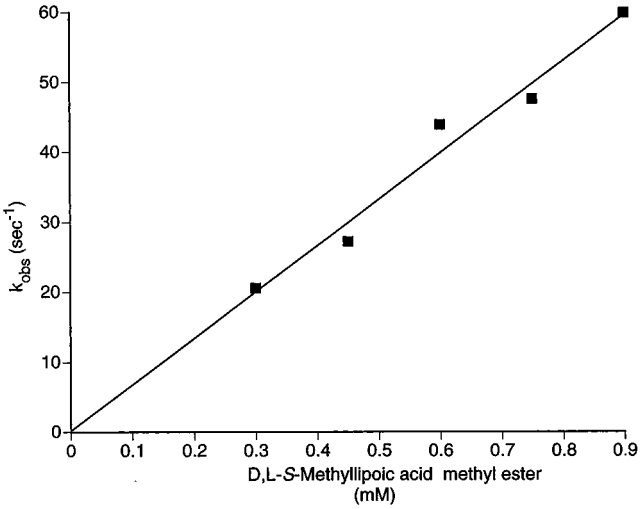


FIGURE 3: Evidence for second-order conditions in the oxidation of the enamine from **1** by D,L-*S*-methyllipoic acid methyl ester.

Table 2: Summary of ^{13}C NMR and ES-MS Data on Compound **6** Reacted with DBU and D,L-*S*-Methyllipoic Acid Methyl Ester

amount added	C13 δ of C2α carbon(ppm)/ assigned structure[a]	ES-MS detected (*m*/*e*)/calculated
9.8 mg **6**	69.509/**6** thiazolium	234.1/234.3
0.5 equiv DBU	193.209/**7** benzaldehyde 69.509/**6** thiazolium	NA
1.0 equiv DBU	189.261/**9** 2-benzoylthiazoline 193.209/**7** benzaldehyde 69.509/**6** thiazolium	NA
1.5 equiv DBU	189.25/**9** 2-benzoylthiazoline	NA
1.5 equiv DBU, then 2.0 equiv **4**	189.87/**9** 2-benzoylthiazoline 187.85/**11** 2-benzoylthiazolium	232.1/232.3

[a] Multiple peaks are listed in order of decreasing intensity.

Presence of DBU. As summarized in Table 2, the C2α carbon has a chemical shift at 69.623 ppm in C2α-hydroxybenzyl-3,4,5-trimethylthiazolium triflate. Addition of 0.5 equiv of DBU led to partial dissociation of the compound to the thiazolium ion and benzaldehyde (whose ^{13}C chemical shift is 193.208 ppm). When an additional equiv of DBU was added, a major new resonance appeared at 189.261 ppm, very likely corresponding to the benzoyl carbon in 2-benzoylthiazoline, which is the stable tautomer of the enamine under these conditions (*16*). In the presence of D,L-*S*-methyllipoic acid methyl ester, a different resonance predominated with a chemical shift at 187.851 ppm. This chemical shift is appropriate for the benzoyl carbon of 2-benzoyl-3,4,5-trimethylthiazolium ion, suggesting oxidation of the enamine to the benzoyl group. A sample taken from the reaction mixture of the enamine derived from C2α-hydroxybenzyl-3,4,5-trimethylthiazolium with D,L-*S*-methyllipoic acid methyl ester gave rise to a major positive ion peak at *m*/*e* of 232.1 in the ES-MS spectrum, consistent with its assignment to 2-benzoyl-3,4,5-trimethylthiazolium ion as the major product. No peak was detected at 341, the *m*/*e* expected for a protonated *S*-benzoyl-*S*-methyldihydrolipoic acid methyl ester, the product expected had the benzoyl group been transferred from the thiazolium ring to the *S*-methyldihydrolipoic acid.

DISCUSSION

Because of the important biological function of lipoic acid in enzymes, especially in the 2-oxoacid dehydrogenase

multienzyme complexes, its chemical properties have been widely studied. Lipoic acid has ring strain in its five-membered ring, and this strain has been invoked to account for its higher reactivity compared to that of linear disulfides (*17*). However, the difficulties in modeling the redox reaction between the enamine and lipoic acid suggested that there might be factors in addition to ring strain controlling the reaction. On the basis of our previous report, in which Hg was used to trap the reduced dihydrolipoate, we were led to mimic an acidic (proton donor) amino acid side chain, namely, to use S-methylated lipoic acid as a model for S-protonated lipoic acid.

A search of the literature indicated that thiosulfonium ions are good electrophiles. As an example, they add to double bonds of alkenes (*18*). Their electrophilic reactions with amines, sulfides, and halides were also reported (*15*). The methylation of lipoic acid was studied by Caserio and her co-workers. From the ^{1}H NMR spectra they concluded that methylation of lipoic acid was nonselective at the S6 and S8 atoms, forming four regio- and stereoisomers in a ratio of 26:11:33:30 (*19*). Caserio and Kim had also suggested that the reactivity of lipoic acid may be related to that of thiolanium ions (*20*).

*Reaction between the Enamine from **1** and **4**.* The isolation and characterization (by ES-MS and NMR) of the tetrahedral adduct is very significant for providing the first direct evidence for the ability of the enamine to be trapped by lipoic acid in a covalent complex prior to acyl transfer, and the observation is certainly consistent with simultaneous bond formation and redox. It is important to point out that such an intermediate had been detected from linear disulfides (*11*), but never from lipoic acid (*9*). On the basis of known reaction of thiosulfonium ions with alkenes, one may question whether D,L-*S*-methyllipoic acid methyl ester could react with the double bonds of the thiazolium ring rather than with the C2α carbon. The *m*/*e* detected by ES-MS cannot exclude this possibility, but the 13 ppm deshielding experienced by the C2α carbon would not be consistent with elimination of the positive charge in, or aromaticity of the thiazolium ring. Rather, the chemical shift change is consistent with replacement of hydrogen by a sulfur at the C2α atom, expected from the reaction of the enamine and D,L-*S*-methyllipoic acid methyl ester. The thiazolium proton chemical shifts also provide evidence against addition to the thiazolium ring carbons: the chemical shifts of the C4- and C5-methyl groups are at 2.422 and 2.461 ppm in the starting material, and in the adduct there are two sets, one at 2.452 and 2.479 and the other at 2.569 and 2.596 ppm. These small chemical shift changes are inconsistent with any reaction taking place on the thiazolium ring.

While one can certainly write an electron transfer mechanism for the formation of the tetrahedral adduct, the observation of only the tetrahedral adduct in the reaction mixture argues against such a mechanism. During electrochemical oxidation of similar enamines, a one-electron mechanism was identified and resulted in the formation of dimers of the enamine (formed at the C2α atom between two cation radicals) as the *only* isolable products. Hence, the absence of any detectable amount of dimer in the reaction mixture is more consistent with a nucleophilic mechanism than with the radical mechanism.

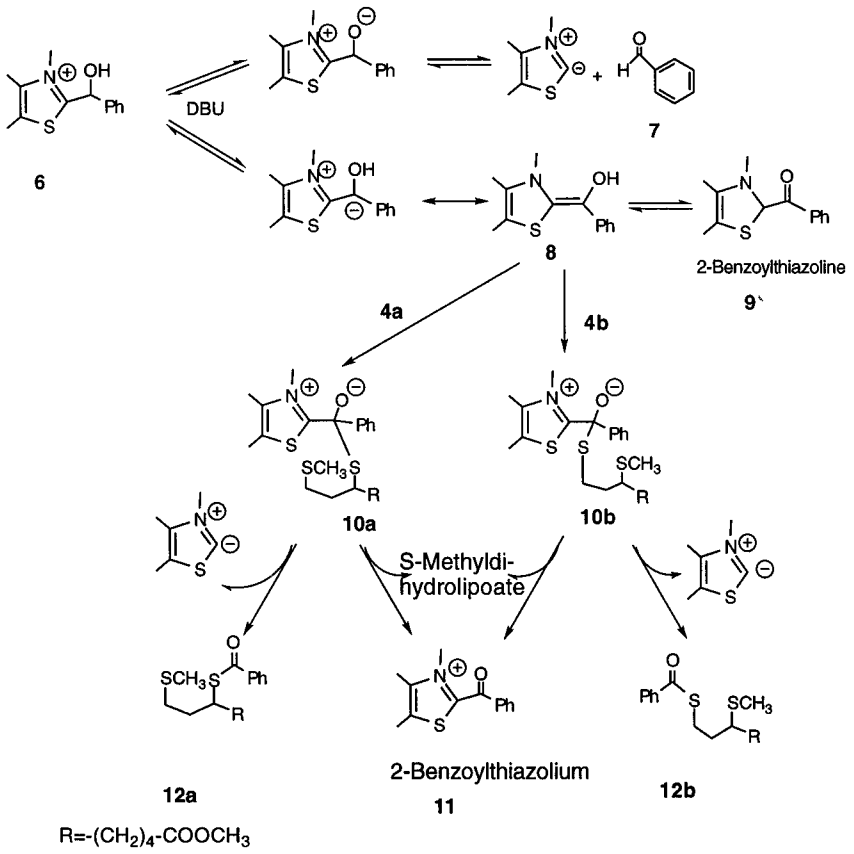
The results on this system lead to the following conclusions: (1) the enamine derived from **1** can be readily oxidized by **4** and leads to the formation of **5**; (2) the reaction most likely takes place by a 2-electron nucleophilic attack; (3) S-methylation accelerates the reaction with the enamine by at least a factor of 10^8. The second-order rate constant measured for enamine oxidation by **4** is 6.6×10^4 M^{-1} s^{-1}, very fast compared to the first-order rate constant of 3.2×10^{-4} s^{-1} measured in Chiu's intramolecular model (*9*). Taking the ratio of the intramolecular to intermolecular rate constants provides an effective molarity of approximately 4.8×10^{-9} M, i.e., this is the concentration of D,L-*S*-methyllipoic acid methyl ester required to match the intramolecular reaction rate between the enamine and 1 M unmodified lipoic acid. The number is all the more impressive when one recognizes that in the previous model the reaction only took place with an added Hg trap, and that in the absence of such a trap, the reaction between the enamine and unmodified lipoic acid has never been achieved either in this lab or elsewhere.

Reaction between C2α-Hydroxybenzyl-3,4,5-trimethylthiazolium Triflate and D,L-S-Methyllipoic Acid Methyl Ester in the Presence of DBU. Unlike with the C2α-methoxybenzyl-3,4,5-trimethylthiazolium case, this time the final reaction product rather than the tetrahedral adduct could be observed. In our experiment carried out in an organic solvent (CH_2Cl_2), 2-benzoylthiazolium ion was the only product observed. While this clearly shows that oxidation of the enamine had taken place, it does not shed any light on the nature of the intermediate or the mechanism. One can speculate that had this reaction also proceeded via the tetrahedral intermediate (**10a** and **10b** in Scheme 3), the partitioning of that intermediate would have to led to (A) to *S*-acyldihydrolipoate (aryl in the model) and regenerated thiazolium ylide; or (B) to the non-productive pair, 2-acylthiazolium (2-aroylthiazolium in this model) plus dihydrolipoate. Since only products resulting from pathway B were observed, the putative tetrahedral intermediate in this reaction did not proceed along the productive pathway. Admittedly, the presence of the methyl group in place of the proton may create unfavorable steric constraints to dissociation of the tetrahedral intermediate to the desired products, and our model used a benzoyl, rather than an acetyl group.

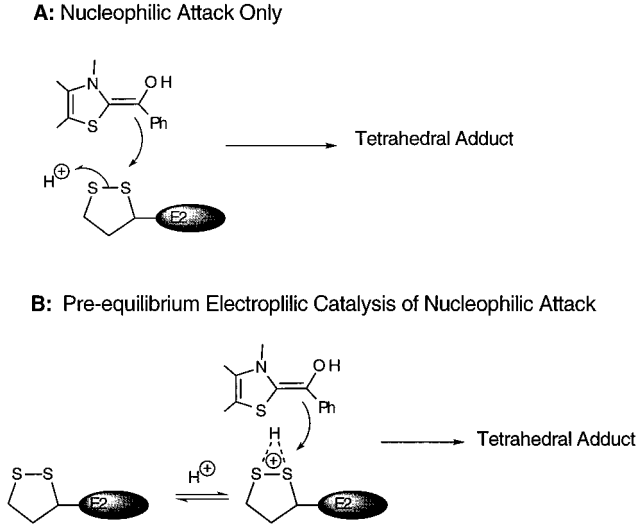
The formation of 2-acyl/2-aroylthiazolium in enzymatic systems has precedent. In a series of studies from Frey's labs, it was shown that 2-acylthiamin diphosphates can be identified from reaction mixtures of 2-oxoacid dehydrogenase multienzyme complexes, perhaps also the result of the unproductive partitioning of the tetrahedral intermediate (*10*). Also, in this lab it had been shown that the linear disulfide 4,4′-dithiodipyridine can trap the enamine produced on both pyruvate decarboxylase and pyruvate dehydrogenase (an assay specific for determination of E1 activity was developed on the basis of this system in ref *21*). Presumably, this reaction also proceeds via a tetrahedral intermediate that collapses to 2-acylThDP and 4-thiopyridine. The indirect evidence for formation of both a tetrahedral intermediate and a 2-acetylThDP is that the chromophore that is generated as a final product represents *N*-(4-thiopyridyl)acetyl amide, rather than the acetic acid thiol ester. The results were interpreted to mean that the nitrogen end of the ambident nucleophile 4-thiopyridine is much better at the deacylation of 2-acylThDP than the sulfur end.

Relationship between the Model Developed and Other Examples of Electrophile-Assisted Reactions at Thiosulfo-

Scheme 3: Proposed Mechanism for the Reaction of the Enamine from **6** with D,L-*S*-Methyllipoic Acid Methyl Ester



Scheme 4: Two Mechanisms for Formation of the Tetrahedral Adduct



nium Centers. There are two distinct mechanisms to rationalize the formation of the tetrahedral adduct (Scheme 4). In pathway A, the enamine attacks at one of the sulfur atoms leading to cleavage of the disulfide bond, followed by protonation of the free thiolate by a nearby proton donor. Pathway B requires the formation of a thiolanium ion in a pre-equilibrium step, followed by nucleophilic attack on it by the enamine and concomitant S−S scission. The second pathway for disulfide bond scission is also termed concomitant electrophilic and nucleophilic catalysis (*22*), or in some instances "push-pull" catalysis, because of initial creation of a highly electrophilic species, followed by the nucleophilic attack. The general acid catalytic pathway for the reduction of lipoate is supported by the early studies of electrophile-assisted disulfide bond scission studied by Kice and Fava. Pre-equilibrium protonation at the sulfur atom was found under acidic conditions, such as in acetic acid with low concentrations of H_2SO_4 (*22*). Kice and Ekman (*23*) studied the disproportionation of disulfides under acidic conditions, their results implied the existence of species $RSS(H^+)R'$, and the results were most consistent with ionic, not free-radical intermediates. Pappas (*24*) carried out ab initio calculations at high levels of approximation (large basis function set) on this issue; the results indicated that, in accord with deductions of Kice and co-workers, the lowest energy pathway involves nucleophilic substitution at sulfur either on the RSSR or on the $RSS(H^+)R'$ species. The previous sluggish model achieved with a mercury trapping reagent also supports such a thiolanium ion mechanism. Sulfur can form a complex with Hg^{2+} because of the high affinity of sulfur for this metal ion, and this metal−thiosulfonium complex also reacts via combined electrophilic and nucleophilic catalysis (*25*).

While we could not locate pK_as for the $RSS(H^+)R'$ species, relevant proton affinity measurements in the gas phase (*26*) suggested the following relative basicities: $CH_3SH < CH_3OCH_3 < CH_3SSCH_3 < CH_3SCH_3$. Although these numbers refer to the gas phase, the size of the groups is small enough so that their relative basicities should parallel the aqueous values. In comparing such values in acid, we place the pK_a of $RSS(H^+)R$ between those of protonated ethyl ether and water, i.e., not lower than −2 to −3 (*27*).

Implications for the Enzymatic Mechanisms. In terms of energetics, the five-membered ring in the thiolanium ion may not be as strained as the one in lipoic acid, since the yellow

color which is characteristic of the strained disulfide bond of lipoic acid was bleached after reaction with methyl triflate. However, protonation on one of the two sulfur atoms converts this sulfur to a better leaving group, and the other sulfur carries a larger positive charge; hence, it is more prone to nucleophilic attack. The second-order rate constant for oxidation of the enamine by **4** is 6.6×10^4 M^{-1} s^{-1}, about 11 times larger than the second-order rate constant (6.1×10^3 M^{-1} s^{-1}) estimated for oxidation of the enamine by isoalloxazine (as a flavin analog for modeling pyruvate oxidase, see ref *8*), i.e., compound **4** is a superior oxidizing agent compared to FAD.

On the basis of the results, we propose that during reductive acylation mediated by the 2-oxoacid dehydrogenase multienzyme complexes there must be a key proton donor that is poised to protonate lipoamide−E2. We further hypothesize that this general acid group resides on the E1 subunit, as the dithiolane of lipoamide is at the terminus of an approximately 3 nm swinging arm that is highly exposed to solvent according to NMR results on the structure of the lipoyl domains (*2*). One can ask how and whether the enzyme could accomplish protonation of such a weakly basic group. Could this thermodynamically unfavorable proton transfer from an enzymic general acid to lipoic acid take place with a rate constant consistent with the catalytic turnover number?

Let us assume that the pK_a of the RSS(H^+)R is −2 (see arguments above), that of the proton donor (say a carboxylic acid) is 4, then ΔpK_a is −6 for the equilibrium in the direction written

$$\mathrm{RSSR} + \mathrm{RCOOH} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \mathrm{RSS(H^+)R} + \mathrm{RCOO^-}$$

Assuming that the rate constant k_r is diffusion controlled ($\sim 10^{10}$ M^{-1} s^{-1}), the k_f (for the $\Delta pK_a = -6$) is 10^4 M^{-1} s^{-1}. This number with a local concentration (effective molarity) of lipoyl−E2 of 10 mM or less, is sufficient to provide a first-order rate constant of 10^2 s^{-1}. This number, in turn, is of the same order as that expected for the turnover number for E1−E2. While the turnover number for PDHc is difficult to obtain accurately given the large number of the various subunits, for both pyruvate oxidase and pyruvate decarboxylase they are about 60 s^{-1}/subunit. That is, for a very reasonable effective molarity of the lipoyl−E2 subunit bound to E1, the protonation of lipoic acid is a possibility. We note that such p*K* perturbations on enzymes are now well-established (*28*, *29*).

In summary, the tetrahedral adduct formed between the enamine/C2α-carbanion and D,L-*S*-methyllipoic acid methyl ester was isolated and characterized for the first time. The rate of the reaction leading to this adduct is very fast (while there is no reaction under similar conditions between the enamine and unmodified lipoic acid) and provides a viable model for the key redox step between the E1-ThDP-bound enamine and lipoamide−E2 and suggests a solution to a long-standing puzzle. The results favor the formation of the tetrahedral intermediate, likely via nucleophilic attack by the enamine and concomitant electrophilic catalysis. The observation of the 2-benzoylthiazolium salt as the product of the reaction between the enamine derived from C2α-hydroxybenzyl-3,4,5-trimethylthiazolium salt and D,L-*S*-methyllipoic acid methyl ester demonstrates oxidation of that enamine as well, and suggests that there may be a further dilemma to be solved by the enzymes to partition the putative tetrahedral intermediate along the productive pathway.

ACKNOWLEDGMENT

Early discussions with Chingfan Chiu are gratefully recalled.

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BI971835Y